A Gata2 intronic enhancer confers its pan-endothelia-specific regulation

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GATA-2, a transcription factor that has been shown to play important roles in multiple organ systems during embryogenesis, has been ascribed the property of regulating the expression of numerous endothelium-specific genes. However, the transcriptional regulatory hierarchy governing Gata2 activation in endothelial cells has not been fully explored. Here, we document GATA-2 endothelial expression during embryogenesis by following GFP expression in Gata2-GFP knock-in embryos. Using founder transgenic analyses, we identified a Gata2 endothelium enhancer in the fourth intron and found that Gata2 regulation by this enhancer is restricted to the endocardial, lymphatic and vascular endothelium. Whereas disruption of three ETS-binding motifs within the enhancer diminished its activity, the ablation of its single E box extinguished endothelial enhancer-directed expression in transgenic mice. Development of the endothelium is known to require SCL (TAL1), and an SCL-E12 (SCL-Tcfe2a) heterodimer can bind the crucial E box in the enhancer in vitro. Thus, GATA-2 is expressed early in lymphatic, cardiac and blood vascular endothelial cells, and the pan-endothelia-specific expression of Gata2 is controlled by a discrete intronic enhancer.

KEY WORDS: Gata2, Endothelium, Cardiovascular, Lymphatic, Enhancer, ETS, SCL, Mouse

INTRODUCTION
In vertebrates, the vascular network is composed of separate blood and lymphatic systems. Although the blood and lymphatic systems are organized in parallel, the blood vasculature develops and is functional prior to lymphangiogenesis. The murine blood vasculature develops from angioblasts that are associated with the blood islands of the yolk sac. This process, known as vasculogenesis, results in the formation of the initial vascular network, which consists of paired dorsal aortae, the cardinal veins, the vitelline artery and vein, and the endocardial tubes. New endothelial cells and vessels are generated later via a process called angiogenesis (Risau, 1997). Further maturation of this new vasculature occurs via pruning of unneeded branches, resulting in the formation of the mature vascular network.

In the yolk sac, the blood islands consist of a thin layer of angioblasts surrounding primitive erythrocytes. Similarly, in the aorta-gonads-mesonephros region – the initial embryonic site of definitive hematopoiesis – hematopoietic stem cells can be detected budding from the endothelium of the dorsal aorta (de Bruijn et al., 2002). Given the close physical proximity of the very earliest hematopoietic and endothelial cells, it has been speculated that they originate from a common progenitor cell, which has been termed the hemangioblast. A number of transcription factors have been shown to play a role in the development of both cell lineages: for example, cloche is required for the formation of endothelial and hematopoietic progenitors in zebrafish (Stainer et al., 1995) and Scl (also known as Tall – Mouse Genome Informatics), which encodes a basic helix-loop-helix transcription factor, was initially shown to be required for hematopoietic development in mice (Robb et al., 1995; Shivdasani et al., 1995). Subsequent transgenic rescue of the hematopoietic defect in Scl-null embryos revealed a requirement for SCL in the remodeling of the yolk sac vasculature (Visvader et al., 1998), and it has since been shown to play a role in vasculogenesis (Patterson et al., 2005), as well as in the migration and morphogenesis of endothelial cells (Lazrak et al., 2004). Transgenic expression of SCL is able to rescue the phenotypic consequences of cloche mutation in the zebrafish, suggesting that Scl functions downstream of cloche (Liao et al., 1998). LMO2, a member of the LIM domain family, is required for primitive erythropoiesis in the embryo; Lmo2 ablation results in death at embryonic day (E) 9.75 secondary to hematopoietic failure (Warren et al., 1994). Analysis of chimeric mice bearing contributions from Lmo2+ embryonic stem (ES) cells revealed that angiogenic remodeling of blood vessels requires Lmo2 (Yamada et al., 2000). Similarly, targeted disruption of the transcription factor Runx1 eliminates definitive hematopoiesis and results in defective angiogenesis and hemorrhaging throughout the CNS (Wang et al., 1996).

The most-widely accepted and experimentally supported model for lymphatic development has proposed that the lymphatic vasculature arises from the blood vasculature (Sabin, 1902; Sabin, 1904; Wigle and Oliver, 1999). Expression of the lymphatic endothelial hyaluronan receptor gene (Lyve1; also known as Xlk1 – Mouse Genome Informatics) at E9.95 in endothelial cells lining the anterior cardinal vein is the first sign that these cells are competent to become lymphatic endothelial cells (LECs). The lymphatic regulatory gene Prox1, encoding a homeobox transcription factor, is expressed several hours later in a subset of Lyve1+ cells in the anterior cardinal vein (Oliver, 2004). Expression of the murine vascular endothelial growth factor receptor 3 gene (Vegfr3, also known as Flt4 – Mouse Genome Informatics), which binds VEGF-C, is detected in blood and lymphatic vessels during early embryogenesis, but becomes largely restricted to lymphatic vessels after E14.5 (Kaipainen et al., 1995).

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Beginning at E10.5, LECs bud and migrate away from the anterior cardinal vein in a polarized non-random manner, and eventually fuse to form primitive lymph sacs from which new LECs sprout and spread into the surrounding tissues and organs (Wigle and Oliver, 1999). Finally, the lymphatic plexus undergoes remodeling and maturation in the terminal stages of lymphatic development (Oliver, 2004; Oliver and Alitalo, 2005; Sabin, 1902; Sabin, 1904). Little is known about the molecular events leading to lymphatic development, but gene-ablation studies in mice and the identification of human hereditary-lymphedema causative genes indicate that Prox1, Vegfc, Vegfr3, Foxc2 and Sox18 are requisites to the process (Wigle et al., 2002; Wigle and Oliver, 1999; Fang et al., 2000; Irrthum et al., 2003; Karkkainen et al., 2000; Karkkainen et al., 2004; Petrova et al., 2004).

GATA factors belong to an evolutionarily conserved family of C4 zinc-finger transcription factors that play demonstrably crucial roles in development. There are six GATA family members in vertebrates, which have historically been subdivided into two subfamilies. GATA-1, GATA-2 and GATA-3 are all important in the development of different hematopoietic lineages - erythroid, hematopoietic progenitor and T-lymphoid, respectively – among many other activities (Pandolfi et al., 1999; Pavly et al., 1995; Tsai et al., 1994). Similarly, GATA-4, GATA-5 and GATA-6 have been shown to be involved in cardiac, genitourinary and multiple endodermal developmental events (Molkentin, 2000; Molkentin et al., 1997; Molkentin et al., 2000; Morrisey et al., 1998).

GATA-2 was originally cloned from a chicken reticulocyte cDNA library (Yamamoto et al., 1990), and was shown to be expressed in a wide variety of tissues, including hematopoietic, neuronal and endothelial cells. Gata2-null mutant embryos die at mid-gestation due to a block in primitive hematopoiesis (Tsai et al., 1994). Further examination of Gata2 gain-of-function and in vitro differentiation of Gata2-ES cells showed that GATA-2 plays a pivotal role in the proliferation of very early hematopoietic progenitors (Briegel et al., 1993; Kitajima et al., 2002; Tsai and Orkin, 1997), underscoring the conclusions from the initial loss-of-function experiments.

Given that many genes involved in hematopoiesis also participate in vascular development and that GATA-2 is strongly expressed in endothelial cell lines, it was originally believed that loss of GATA-2 function would result in vascular defects. Adding further to this expectation was early evidence that many genes that appeared to be crucial for endothelial development and function are regulated via GATA-binding sites (Dorfman et al., 1992). For example, GATA sites are involved in the regulation of the endothelium-specific genes preproendothelin (immature form of EDN1) (Dorfman et al., 1992; Yamashita et al., 2001), Pecam1 (Gumina et al., 1997), Vegfr2 (Kappel et al., 2000; Minami et al., 2004), eNOS (also known as Nos3 – Mouse Genome Informatics) (German et al., 2000) and Icam2 (Cowan et al., 1998). Mutation of a GATA-binding site in the Vegfr2 endothelium-specific enhancer completely abolished its activity in transgenic reporter assays, indicating that Vegfr2 expression is dependent on GATA activity in vivo (Kappel et al., 2000). Surprisingly, however, the analysis of Gata2-null embryos failed to reveal any obvious defects in the vasculature at the time of their early embryonic demise (~E10) (Tsai et al., 1994), leaving the role for GATA-2 in endothelial function undefined.

To begin to investigate the role of GATA-2 in endothelial function, we systematically examined GFP expression in the developing vasculature of Gata2-GFP knock-in embryos during embryogenesis. We found that GFP was expressed in cells lining arterial and venous vessels formed during vasculogenesis and angiogenesis, and that its expression continued postnatally. We also observed GFP expression in budding LECs during early lymphatic development, as well as in postnatal lymphatic vessels. We then functionally identified an endothelium-specific enhancer in Gata2 intron 4 that could regulate the expression of a cis-linked reporter transgene in cardiovascular and lymphatic endothelial cells. Additionally, we found, using site-specific mutagenesis, that the potency of the minimal endothelium-specific enhancer is crucially dependent on an E box (CANNTG) motif. By contrast, disruption of three ETS-binding sites quantitatively reduced, but did not abolish, enhancer activity. Prior experiments showed that SCL activation is required for elaboration of the vasculature, and we demonstrate that SCL-E12 (E12 is also known as TCFE2A – Mouse Genome Informatics) heterodimers bind with high affinity to this crucial enhancer E box in vitro. Altogether, these data implicate ETS family members and SCL as in vivo activators of endothelium-specific Gata2 transcription.

**MATERIALS AND METHODS**

**Transgenic mice**

Wild-type CD1 mice were mated with Gata2-GFP knock-in heterozygous mice, which had GFP inserted (in frame) at the translation initiation codon in Gata2 exon 2 (Suzuki et al., 2006). Embryos were harvested at the times indicated in the text and figure legends, and processed for immunostaining as previously described (Khandekar et al., 2004). Reactivity to rabbit anti-GFP (1:1000; Molecular Probes), rat anti-PECAM (1:200; Pharmingen), goat anti-VEGFR3 (1:20; R&D Systems), rabbit anti-PROX1 (1:800; Covance) and rabbit anti-LYVE1 (1:400; Upstate) antibodies was detected using the appropriate fluorochrome-conjugated secondary antibodies, as indicated in the figure legends. Digital images were recorded as previously described (Khandekar et al., 2004).

For founder transgenic analyses, expression constructs were purified for microinjection into fertilized ova as previously described (Khandekar et al., 2004). At the indicated times, embryos from foster mothers or a Gata2 YAC d162z genomic line were harvested for X-gal staining and PCR genotyping as previously described (Zhou et al., 1998). Transgenic embryos were photographed as whole-mount or cryosectioned specimens as described previously (Zhou et al., 1998).

**Expression-plasmid construction**

For microinjection, plasmid GR22-lacZ was digested with different restriction enzymes (see legend to Fig. 3) (Zhou et al., 2000). Other Gata2 fragments examined here were cloned 3’ to the herpes simplex virus (HSV) thymidine kinase (TK) gene promoter in TKβ (Clontech) to mimic their natural position in the Gata2 locus. To generate TKBXβ, a 2.9 kbp BamHI-SalI fragment from plasmid GR22 was first subcloned into pBluescript II (Strategene) and then excised with XbaI before re-cloning into XbaI-digested TKβ. To generate TKSXβ, TKBXβ was treated with SpeI-SfiI and T4 DNA polymerase before self-religation. To construct TKAAβ, a 460 bp AlwNI-Apal fragment was excised from plasmid GR22 and treated with T4 DNA polymerase before being cloned into TKβ, which had been treated sequentially with XbaI and with Klenow polymerase. For microinjection, TKANβ was generated from TKAAβ by NcoI restriction-enzyme digestion. To delete the internal AlwNI-Apal fragment, a plasmid subclone containing the 1.2 kbp SfiI-Xhol Gata2 intron 4 was treated with AlwNI-Apal and T4 DNA polymerase before self-religation. The resultant 0.8 kbp SfiI-Xhol fragment was cloned into TKβ to generate TKSXAAβ. To clone the vascular endothelium-specific (VE) enhancer into TKβ (thereby generating TKVEβ, Fig. 5), primers Endocons(f) and Endocons(r) containing an engineered XbaI site (5'-ggtctagaCCATGGAGTCACCTATACTGTG-3') and 5'-ggtctaga- AACTGATCGAAGGTGCTCTG-3', respectively) were used to generate a 167 bp amplicon (defined by the arrows in Fig. 4A, Fig. 5A), which was verified by sequencing.

To mutate the E box in the VE enhancer, oligonucleotide-based PCR mutagenesis was performed to introduce mutations (from 5’-CAGTCTG-3’ to 5’-ACCCG-3’; mutations are lowercase) that had been shown to eliminate SCL binding in gel shift assays (Kappel et al., 2000). Primers EcSLmut(f) (5’-CCGACTGCCACGACCACGCG-3’) and Endocons(r) (shown above) were used to generate a 3’ fragment using GR22 plasmid as template in a PCR reaction.
Similarly, EcSCLmut(r) (5′-CGGCTGcggTGCTCGG-3′) and Endocons(f) were used to generate a 5′ fragment. The resultant amplicons were gel-purified and pooled as templates in a PCR reaction using Endocons(f) and Endocons(r) as primers. The gel-purified PCR products were sequenced to verify incorporation of the mutation and were then digested with XbaI site of TK

\[<\text{mutation}>\] and then cloned into the TKVE enhancer, either the first two or all three ETS-binding motifs were digested with XbaI site of TK

\[<\text{mutation}>\] andEndocons(f) to generate TKVE enhancer, which was transfected or transfected with EF-1α promoter-directed SCL cDNA alone or with a CMV promoter-directed E12 expression plasmid. Either no extract or nuclear extract (35 μg) was added to binding buffer containing 20 mM HEPES (pH 7.9), 1 mM MgCl2, 0.5 mM DTT and 37.5 ng/μl poly(dI-dC) at 4°C. Unlabeled oligonucleotides (20- or 200-fold molar excess; wild type (5′-TCCGACATCTGACGGCGGT-3′; E box underlined) or mutant (5′-TCCGACACcAcGGCGGGCT-3′; E box underlined, mutated nucleotides lowercase) or antibodies (Rodriguez et al., 2005) were added as indicated in the legend to Fig. 5. After 1 hour of pre-incubation, 2 μl (2×105 cpm) of radiolabeled wild-type oligonucleotide probe was added to each sample and incubated for an additional 30 minutes. All samples were fractionated by electrophoresis on neutral 6% TBE/polyacrylamide gels. After electrophoresis, the gels were dried and recorded using a PhosphorImager (Molecular Dynamics).

**RESULTS**

Early, pan-endothelial expression of transcription factor GATA-2

Because the expression of GATA-2 had been reported in several endothelial cell lines (Dorfman et al., 1992; Umetani et al., 2001), we first investigated whether or not Gata2 was expressed in all endothelial cells in vivo by analyzing GFP staining in the vasculature of Gata2-GFP knock-in heterozygotes (Suzuki et al., 2006). In whole-mount E9.5 embryos, robust GFP fluorescence was monitored by direct fluorescence (A), indirect immunofluorescence (B-D,F-R) or light (E) microscopy. (A) Robust GFP fluorescence is visualized in the heart and the dorsal aorta, a vessel formed by vasculogenesis (arrowhead), of a whole-mount embryo orientated with its head (not shown) facing to the left, towards the tail bud. (B-D,F) Transverse embryonic cryosections were stained for GFP using Alexa Fluor 488-conjugated secondary antibody. GFP immunoreactivity was detected in the intersomitic vessels (B, arrowheads) in the tail region of an embryo, in the endothelia lining the aortic sac (C), in the thin-walled umbilical vein and thick-walled umbilical artery (E,F), and in the endocardium of the heart ventricle (C), as well as in the vessels that begin to invade the neural tube, a typical example of sprouting angiogenesis (D). (E) A phase-contrast image of F (G-L) The intersomitic vessels and the aorta in the tail region of an embryo co-stained for GFP (G,J) or PECAM (H,K) antigens using Cy3- or Alexa Fluor 488-conjugated secondary antibodies, respectively. Coincidence of anti-PECAM and anti-GFP staining demonstrates that Gata2 is expressed in endothelial cells (I, L). Boxed areas in G-I are magnified in J-L. (M,N) Transverse embryonic cryosections were stained for GFP (N) or VEGFR3 (M) using CY2- or CY3-conjugated secondary antibodies, respectively. Clustered cells in the vicinity of the anterior cardinal vein expressed both VEGFR3 and GFP (arrowheads). Notice that, although GFP immunofluorescence was detected strongly in endothelia of the dorsal aorta and cardinal vein, both of these blood vessels stained only weakly, in comparison to LECs, with anti-VEGFR3 antibody. (O) An adjacent section was co-stained with anti-PROX1 and anti-VEGFR3 antibodies using Cy2- or Cy3-conjugated secondary antibodies, respectively. Notice that VEGFR3-positive cells displayed anti-PROX1 nuclear staining (arrowhead), thus confirming their LEC identity. (P-R) P1 postnatal intestines and mesentery were sectioned and stained for VEGFR3 (P) and GFP (Q) expression as described above. Coincidence of staining in lymphatic vessels (arrowheads) is distinct from blood vessels that stained only for GFP (arrows). The nuclei in panels L, O and R were co-labeled with DAPI, h, heart; tb, tail bud; as, aortic sac; ven, ventricle; mv, mesencephalic vesicle; uv, umbilical vein; ua, umbilical artery; cv, cardinal vein; da, dorsal aorta.
detected in the dorsal aorta, a vessel formed by vasculogenesis, as well as in the developing heart (Fig. 1A). In transverse cryosections of E10.5 embryos, GFP-immunopositive cells were seen lining the intersomitic vessels (Fig. 1B, arrowheads), the aortic sac and ventricular endocardium (Fig. 1C), and the developing neural tube (Fig. 1D), a site of intense sprouting angiogenesis. These data showed that GATA-2 is abundantly expressed in vessels formed during primary vasculogenesis and angiogenesis.

To determine whether Gata2 was expressed differentially in veins and arteries, we analyzed the umbilical cords of E18.5 embryos, in which a clear morphological distinction can be made between umbilical vein and artery (Fig. 1E). Although there was an apparent difference in the fluorescence intensity, GFP immunoreactivity could be readily visualized in both blood vessels (Fig. 1F). The differential GFP staining might be due to a genuine difference in expression level, or simply due to a difference in the optical density resulting from the greater surface area of the vein.

To verify that the GFP expression was indeed endothelium-specific, we performed co-immunostaining with antibodies directed against GFP and PECAM, an endothelial cell-specific cell-adhesion molecule. When E10.5 embryonic cryosections were stained with an anti-GFP antibody and a CY3-conjugated secondary antibody, vascular structures in the tail bud region stained strongly (Fig. 1G,J). When the same sections were co-stained for the detection of PECAM antigen using Alexa Fluor 488-conjugated secondary antibody (Fig. 1H,K), the anti-GFP and anti-PECAM immunofluorescence were completely coincident (Fig. 1I,L), verifying that Gata2 is expressed quite specifically in vascular endothelial cells.

To investigate whether Gata2 is expressed in the lymphatic vasculature, serial transverse cryosections of an E11.5 Gata2^lacZ^ embryo were stained with anti-VEGFR3 (Fig. 1M) or anti-GFP (Fig. 1N) antibodies. GFP fluorescence was detected strongly in the cardinal vein and dorsal aorta, as well as in clustered cells lying near the anterior cardinal vein (Fig. 1N, arrowheads). However, VEGFR3 immunoreactivity was most pronounced in scattered cells lying near the cardinal vein, from where LECs initially sprout (Fig. 1M, arrowheads). To confirm further that the GFP^+/VEGFR3^+ cells were indeed LECs, a second section was co-stained for the LEC-specific markers VEGFR3 and PROX1 (Fig. 1O). Nuclear anti-PROX1 and cytoplasmic anti-VEGFR3 immunofluorescence co-localized in the same cell population, which was located near the anterior cardinal vein (Fig. 1O, arrowhead). Similarly, GFP-expressing cells were identified in the blood and lymphatic vasculatures of the intestine and the mesentery (Fig. 1Q), and the skin (data not shown) of a postnatal day 1 (P1) pup. Expression of Vegfr3 (Fig. 1P), which is restricted to LECs after E14.5, co-localized with a subset of GFP-positive cells (Fig. 1R, arrowhead). Similar results were obtained with PROX1 and LYVE1 immunostaining (data not shown).

We conclude that Gata2 is a pan-endothelial marker that is expressed early in lymphatic, vascular and endocardial endothelial cells. In the blood vasculature, it is indiscriminately expressed in blood vessels that are formed during vasculogenesis and angiogenesis, as well as in the arterial and venous branches of the embryonic vascular system. In the cardiovascular and lymphatic systems, Gata2 expression persists postnatally.

**A transgenic YAC recapitulates endogenous Gata2 expression in the vasculature**

We previously reported that a 271 kbp Gata2 yeast artificial chromosome (YAC) transgene, containing sequences from −198 to +73 kbp (with respect to the translation initiation site) of the Gata2 locus was capable of rescuing the hematopoetic failure that is the underlying cause of the early embryonic lethality in homozygous Gata2 mutant embryos (Khandekar et al., 2004; Zhou et al., 1998). When we re-examined the β-galactosidase (β-gal) staining in the developing vasculature of E8.5 transgenic embryos bearing the same (d16) YAC, but tagged with lacZ (Zhou et al., 1998), X-gal staining was very prominent in the heart tube (Fig. 2A) and, by E10.5, in the aorta and the endocardium (Fig. 2B). By E12.5, lacZ expression was pronounced throughout the vascular system of the yolk sac and in the embryo proper (Fig. 2C-E), although the staining in the umbilical vein appeared to be fainter than in the umbilical artery (Fig. 2E, uv and ua, respectively). This differential staining was reminiscent of the differential GFP intensity of expression observed earlier in the umbilical vein and artery of Gata2-GFP knock-in heterozygotes. Furthermore, YAC d18Z (−40 to +73 kbp) – a smaller, 5′-deletion derivative of d16Z (Zhou et al., 1998; Zhou et al., 2000) – displayed an identical vascular lacZ pattern in transgenic embryos (data not shown). This led to the tentative conclusion that the regulatory element(s) directing Gata2 endothelial expression lay within the boundaries of these YACs.

**Localization of a Gata2 endothelium-specific enhancer**

While investigating Gata2 activity in the developing nervous system previously, we generated the plasmid GR22-lacZ, which contains 20 kbp of the Gata2 genomic sequence (from −9 kbp to slightly

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**Fig. 2. YAC d16Z contains Gata2 endothelium regulatory sequences.** (A-E) Embryos at E8.5 (A), E10.5 (B) and E12.5 (C-E) bearing the Gata2 d16 lacZ-tagged yeast artificial chromosome (Zhou et al., 1998) were stained for β-galactosidase activity as whole-mount (A,C-E) or cryosectioned (B) specimens. Strong lacZ expression was observed in the developing heart tube (arrowhead; A), in the aorta and the endocardium (ec; B), in the remodeled vasculature of the yolk sac (C), and in the embryo proper (D,E), as well as in the umbilical vessels (E), where the staining superficially appeared to be weaker in the umbilical vein (uv) than in the umbilical artery (ua).
beyond exon 6, with respect to the translational start site) with a lacZ reporter gene inserted in frame at the initiation codon in exon 2 (Zhou et al., 2000). Plasmid GR22-lacZ was separately digested with different restriction enzymes in order to test, by founder transgenic analysis, overlapping fragments for the presence of an endothelium-specific enhancer. Both Xho-I-SalI and KpnI-KpnI fragments reproduced the same endothelium-restricted activity in the majority of lacZ transgene-positive embryos (9/10 and 5/5, respectively; Fig. 3A,B and data not shown) (Zhou et al., 2000). Most instructively, GR22-lacZ KpnI-SflI transgenics displayed a complete loss of endothelial X-gal staining (0/7 embryos; Fig. 3C). Hence, the Gata2 endothelium-specific enhancer activity could be tentatively localized to within a 1.8 kbp SflI-SalI interval in the Gata2 fourth intron.

Next, we tested various fragments in the TKβ vector to assess whether they could function as classical enhancers to drive cardiovascular endothelium-specific expression. When a 2.3 kbp BamHI-XbaI (BX) or a 1.2 SflI-XbaI (SX) fragment was tested in transgenic founders, each retained endothelium-specific enhancer activity (Fig. 3D and data not shown). Discrete lacZ staining was evident in the endothelial cells lining the aorta and heart (Fig. 3E,F), the blood vessels of the yolk sac (Fig. 3G), the umbilical artery and vein (Fig. 3H,I), and the vascular network of the postnatal brain (Fig. 3J) of TKSXβ transgenic animals.

Furthermore, a subset of β-gal+ cells in and surrounding the anterior cardinal vein (Fig. 3K,L, cv) of an E10.5 TKSXβ transgenic embryo stained with the LEC-specific marker LYVE1 (Fig. 3K,L, arrowheads), indicating that the endothelium-specific enhancer is also active in the lymphatic endothelial lineage. Thus, the 1.2 kbp SX fragment was able to function as a classical enhancer and directed lacZ expression throughout the entire developing vasculature, recapitulating the full extent of endogenous Gata2 expression in the endocardial, the blood and the lymphatic vascular systems.

We tested next a series of smaller constructs (Fig. 4A) to establish the boundaries of a minimum enhancer element required to achieve the Gata2 endothelium-specific expression pattern. Deletion of the 1.2 kbp SX fragment from only the 3’ end (in TKSXβ) or from both termini (in TKAAβ) did not alter the cardiovascular endothelium-specific expression in E10.5 transgenic embryos (Fig. 4B,C). By contrast, deletion of the internal AflwNI-ApoI (AA) 460 bp fragment from the SX enhancer fragment eliminated all endothelial enhancer activity (0/15; Fig. 4A,D). Thus, the minimal endothelium-specific enhancer as defined by the AA restriction fragment is sufficient for endothelium-specific Gata2 enhancer activity. Anti-LYVE1 immunostaining of E10.5 TKAAβ transgenic embryos demonstrated that the minimal endothelium-specific enhancer remained active in LECs (data not shown).
Identification of key regulatory motifs within the Gata2 minimal endothelium-specific enhancer

Regulatory elements are thought to diverge more slowly than sequences that surround them (Loots et al., 2000). Comparison of the mouse Gata2 460 bp endothelium-specific sequence to that of the human sequence demonstrated that a 355 bp region within the AA fragment displayed 96% sequence identity, as well as a nearby 58 bp region that harbored 96% identity (data not shown). This extreme degree of evolutionary sequence conservation strongly implies an associated functional significance. Analysis of the 460 bp element using MatInspector 2.2 (Quandt et al., 1995), which uses the consensus transcription factor-binding motifs from the TRANSFAC database, identified a number of candidate regulatory motifs that might bind to this enhancer (Fig. 5A). A closer examination of this restriction fragment showed an unusual clustering of binding sites within a central 167 bp core region (as delineated by the two convergent arrows shown in Fig. 4A and Fig. 5A). Interestingly, the 3’ terminus of this region corresponded closely to the RsrII site that was identified previously as defining the 3’ functional boundary of endothelium-specific activity (Fig. 4A,B).

The mouse 167 bp endothelium-specific fragment (called VE) bearing the highest human-mouse identity was cloned into TKβ to test its ability to recapitulate endothelial expression. Analysis of transgenic founders showed that TKVEβ was able to confer endothelial expression in a range of vascular tissue (14/17; Fig. 5B) that did not differ from the TKAAβ construct. Strikingly, however, none of these embryos (0/17) exhibited endocardium-specific β-gal staining (Fig. 5B, arrow). We surmise from these data, in conjunction with data presented earlier (Fig. 4), that the endothelium-specific activity is largely contained within the 290 bp AlwNI-RsrII fragment of Gata2 intron 4 and that the 5’-most 155 bp of the AlwNI-RsrII fragment are required for Gata2 expression in the endocardium while the adjoining 3’ 167 bp can autonomously direct transgene expression in blood endothelia (Fig. 4A and Fig. 5A).

Fig. 4. Fine localization of the Gata2 endothelium-specific enhancer. (A) Schematic illustrations of transgenic constructs (TKSXβ, TKSρβ, TKAAβ, TKSXΔAAβ and TKANβ) used to functionally localize the Gata2 endothelium-specific enhancer element. Sub-fragments of Gata2 intron 4 were individually cis-linked to a TK promoter-lacZ reporter gene. The positions of relevant restriction enzyme sites (AlwNI, A; Apal, Ap; Ncol, N; RsrI, R; SfiI, S; XbaI, X) and the restriction fragment lengths (in bp) are indicated. The numbers on the right indicate the number of embryos with cardiovascular β-gal staining/total number of transgene-positive embryos. The arrows represent the positions of the primer pairs used to amplify the 167 bp VE enhancer (see Fig. 5). (B-E) E10.5 embryos bearing TKSρβ (B) or TKAAβ (C) transgenes showed widespread endothelial β-gal staining, whereas the TKSXΔAAβ (D) and TKANβ (E) transgenic embryos were devoid of endothelial X-gal accumulation. In the latter embryos, only ectopic β-gal activity was observed.

Fig. 5. Identification of a crucial E box for Gata2 vascular endothelium enhancer activity. (A) Consensus binding motifs for candidate regulatory effectors within the evolutionarily conserved 460 bp AlwNI-Apal endothelium-specific enhancer sequence are highlighted. The 167 bp minimal vascular endothelium-specific (VE) enhancer in TKVEβ (B) was generated using the PCR primer pairs indicated by the two convergent arrows. The italicized sequences correspond to the radiolabeled probe used for EMSA studies (see Fig. 6). (B) TKVEβ recapitulates widespread vascular (14/17), but not endocardial (0/17, arrow), endothelial lacZ expression in E10.5 transgenic embryos. (C) Simultaneous mutation of all three ETS1-binding consensus sites (A) in TKVEβmEts1,2,3 resulted in far fewer (3/25) transgenic embryos that displayed vascular endothelium-specific lacZ expression. (D) Disruption of the single SCL-binding site (A) in TKVEβmScl completely abrogated vascular endothelium-specific X-gal accumulation (0/24). (E-G) Transverse sections through the hearts of E10.5 embryos bearing TKSρβ (E; Fig. 4B), TKAAβ (F; Fig. 4C) or TKVEβ (G; Fig. 5B) transgenes. Notice the conspicuous absence of X-gal staining in the endocardium of the ventricular chamber of the TKVEβ embryo.
An SCL-E12 heterodimer avidly binds to the E box motif in the Gata2 VE enhancer

Because SCL had been shown previously to be essential for endothelial differentiation (Visvader et al., 1998), we wished to determine whether SCL could bind to the E box in the VE fragment. To do so, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts from 293T cells transfected with either SCL, or SCL plus E12, expression vectors. Incubation of radiolabeled E box oligonucleotide probes with nuclear extracts containing SCL-E12, but not SCL alone, resulted in a low-mobility complex (Fig. 6, lane 4), which could be specifically competed by the addition of an excess of unlabeled E box oligonucleotide (Fig. 6, lanes 5, 6), but not by mutant E box oligonucleotide (Fig. 6, lanes 7, 8). The binding specificity and protein identity were confirmed by showing that addition of an anti-SCL antibody (Fig. 6, lanes 9, 10), but not control IgG (Fig. 6, lane 11), significantly reduced EMSA complex formation. Thus, the crucial E box motif in the Gata2 VE enhancer can be strongly bound by the basic helix-loop-helix transcription factor SCL.

DISCUSSION

In summary, the data presented here delimit the boundaries of a functionally defined 460 bp Gata2 fourth intron endothelium-specific enhancer element that is capable of autonomously directing reporter gene expression in vivo in vascular, endocardial and lymphatic endothelial cells, thus precisely mimicking endogenous GATA-2 expression. Given the paucity of molecular markers and tools unique to the LEC lineage, the Gata2 endothelium-specific enhancer may serve as a useful additional marker in the lymphatic field. Both ETS- and E box-binding sites contribute to the potency of the VE enhancer, thus implicating Ets family member(s) and SCL as candidate regulatory effectors of Gata2 vascular endothelial expression.

Identification of Gata2 pan-endothelium-specific enhancer

The existence of an endothelial enhancer for Gata2 raises several intriguing questions about its function. Earlier genetic data indicated that GATA-2 plays a crucial role in early hematopoietic development (Tsai et al., 1994) and is indeed capable of specifically marking hematopoietic stem cells (Suzuki et al., 2006). Given that the earliest hemangiogenic cells are closely related to the endothelial lineage (Choi et al., 1998), one might speculate that this endothelial enhancer could also target a subset of hematopoietic cells that are generated from the aortic endothelium. Suggestively, we identified lacZ-positive cells that appear to be “budding” from the aortic wall (Fig. 3E, arrow). Whether these cells are bona fide hematopoietic cells or simply endothelial cells being sloughed into the aorta is yet to be determined. However, the existence of an endothelium-specific enhancer of Scl, which also marks hematopoietic progenitors (Gottgens et al., 2004), suggests that the Gata2 intron 4 enhancer identified here may play some role in the appropriate regulation of Gata2 in hematopoietic progenitors as well, particularly in light of the recent observation that this enhancer is active in definitive erythroid cells (Grass et al., 2006) (see below).

We report here that Gata2 is also expressed at the earliest stage of lymphangiogenesis, when LECs bud from the anterior cardinal veins at mid-gestation, and that it continues to be expressed in the postnatal lymphatic vasculature. Although the initial budding of PROX1+ LECs appeared to be normal in GATA-2-deficient embryos (data not shown), it remains to be determined whether lymphatic development after E10, the nominal time of death of Gata2-null embryos, continues unperturbed.

To ascertain whether the 5′-most 155 bp fragment was sufficient for endothocardium-specific reporter gene activation, we tested the AlwNI-NcoI fragment in the context of TKβ (Fig. 4A), and found that, of 11 recovered transgenic embryos, none displayed endocardial X-gal staining despite exhibiting variably ectopic X-gal staining (Fig. 4A,E). We conclude that the 5′-most 155 bp of the endocardium-specific enhancer alone is incapable of independently directing Gata2 endocardium expression.

The Ets family of transcription factors have been shown to play crucial roles in vascular development (Ayadi et al., 2001; Wang et al., 1997), and have also been reported to play prominent roles in endothelium-specific enhancer activity (Gottgens et al., 2002; Kappel et al., 2000). The existence of three ETS-binding sites within the 167 bp Gata2 VE enhancer suggested that one or multiple Ets family members might modulate its activity. To directly address this hypothesis, the three ETS-binding sites were individually mutated from CGGA to CGgc, a mutation that was previously shown to eliminate ETS-factor binding (O’Reilly et al., 2003). Mutation of either two or all three ETS-binding sites reduced the overall number of embryos displaying weak, albeit non-specific staining (1/12 and 3/25, respectively; data not shown and Fig. 5C).

Next we determined whether the single E box motif present in the 167 bp VE enhancer is important for its overall activity. A mutation (5′-CATCTG-3′ to 5′-CAcccG-3′) that was previously shown to eliminate SCL binding (Kappel et al., 2000) was incorporated into the TKVEβ plasmid. Among the 24 recovered TKVEβmScI transgenic embryos, none displayed endothelial β-gal staining (e.g. Fig. 5D). This lack of staining was presumably not due to the effect of transgene integration site, because some of the transgenic embryos (9/24) exhibited ectopic staining in the spinal cord or head. These data demonstrate that the single Gata2 intron 4 E box exerts a profound effect on the activity of the VE enhancer and, by extension, in the regulation of Gata2 expression throughout the blood endothelium.

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In the blood vasculature, the lack of any reported phenotype in Gata2 
embryos led to the initial conclusion that functional redundancy of other GATA family members, including GATA-4 and GATA-6, may compensate for any lack of GATA-2 in the endothelium. Another equally plausible possibility is that Gata2 mutant embryos simply die too early (~E10) (Tsai et al., 1994) to generate a robust vascular phenotype. Based on the analysis of Gata2-lacZ knock-in mice, Gata3 expression in the endothelium does not appear to be widespread, but this closely related GATA family member does seem to be spuriously active (our unpublished data). Gata4, which plays a crucial role in heart development, does not appear to be expressed in mature endothelial cells (Umetani et al., 2001), although it is expressed in endothelial progenitors (Hatzopoulos et al., 1998). Gata6 is expressed in both endothelial precursors as well as in mature endothelial cells (Hatzopoulos et al., 1998; Umetani et al., 2001), making GATA-6 a prime candidate for a possible Gata2-complementing endothelium activity. Whereas Gata4 and Gata6 heterozygotes are normal, compound heterozygotes display cardiovascular defects (Xin et al., 2006). Notably, these embryos displayed a less intricate weave, which was disorganized, of the cranial and intersomitic vasculature, as well as hemorrhaging. The recent generation of a Gata6 conditional loss-of-function allele (Sodhi et al., 2006) should now permit exploration of cell autonomous GATA-6 involvement, if any, in endothelial development.

**Role of Ets family transcription factors in Gata2 endothelium-specific enhancer activity**

Within an initial functionally defined restriction fragment describing the Gata2 endothelial enhancer, we subsequently identified a 167 bp core enhancer that was sufficient to recapitulate vascular endothelial expression. The existence of three putative Ets family-member-binding sites implicated a role for these factors in the control of this VE enhancer. The Ets family of transcription factors have been shown to play an important role in vascular development in vivo (Sumanas and Lin, 2006) and have been shown to be functionally important in the activation of a number of endothelial-specific enhancers, including Scl (Gottgens et al., 2004), Tie2 (also known as Tek) (Minami et al., 2003) and Flk1 (also known as Kdr) (Elvert et al., 2003). Furthermore, disruption of Tel (also known as Etv6), one Ets family member, results in defective yolk sac angiogenesis (Wang et al., 1997), suggesting that TEL plays an important role in vascular remodeling. However, targeted mutation of other ETS factors has not revealed vascular deficiencies, suggesting that these factors may either play no role or may also be functionally redundant in endothelium development.

In the Gata2 VE enhancer defined here, mutations predicted to disrupt ETS-binding sites significantly attenuated enhancer activity, as indicated by the number of, and X-gal-staining intensity in, transgenic embryos displaying endothelial β-gal staining. However, the weak staining pattern detected in these embryos appeared to remain endothelium-specific, indicating that the ETS-binding sites are not essential for the tissue specificity of the enhancer, but rather may serve to augment its overall potency. Because this cis mutation has been shown in a similar assay to eliminate DNA binding for some members of the family (O’Reilly et al., 2003), we cannot rule out the possibility that the mutation does not abolish the binding of the multiple Ets family members that are expressed within the endothelium (Lelievre et al., 2001). Additionally, the heterogeneity of ETS-binding sites suggests that some family members may be able to bind to other sequences within the enhancer, enabling endothelial activation despite mutations within canonical high-affinity binding sites.

**An E box-binding factor is required for Gata2 endothelial enhancer activity**

The transcription factor SCL has been shown to play crucial roles in both hematopoiesis (Shivdasani et al., 1995) and vascular development (Patterson et al., 2005; Visvader et al., 1998), leading to the speculation that SCL may be important for the ontogeny of the hemangioblast. A comprehensive analysis of the transcriptional regulation of Scl has identified several tissue-specific enhancers that are required for its appropriate expression (Barton et al., 2001; Gottgens et al., 2002; Sinclair et al., 1999). Interestingly, the enhancer specific for hematopoietic progenitors has GATA sites that are crucial for Scl enhancer activity in vivo. The factor responsible for binding to these sites in hematopoietic cell lines appears to be GATA-2, suggesting that GATA-2 is responsible for activating Scl in early hematopoiesis. However, there is no GATA-binding site in the endothelial enhancer of human SCL, although it remains possible that a GATA factor is acting without directly binding to DNA (Gottgens et al., 2004). The data presented here are consistent with the possibility that Gata2 and Scl encode reciprocally reinforcing activators in these developmentally related tissues, although other interpretations are clearly not excluded from the data presented.

To assess the relationship between Scl and Gata2 in the endothelium, we mutated the single E box present within the 167 bp Gata2 VE enhancer. Scl has previously been shown to be regulated by GATA factors in both the CNS (Sinclair et al., 1999) and hematopoietic progenitors (Gottgens et al., 2002), suggesting that the nature of the epistatic relationship between these two factors may be dependent on the specific tissue in question. However, these data also underscore the point that the functions of these two factors are often intimately intertwined during development. Lending further credence to this point is the evidence that Scl expression in the endothelium is crucially dependent on Ets-family activity (Gottgens et al., 2004). The endothelial enhancer of Scl contains five ETS-binding sites that are required for the activity of the enhancer in trans-activation assays. Given that both Scl and Gata2 appear to be regulated by ETS factors in the endothelium, we surmise that ETS, SCL and GATA-2 together constitute a regulatory circuitry wherein, in the simplest scenario envisaged, ETS factors activate Scl, and ETS and SCL then cooperate to activate Gata2 in endothelial cells. Similarly, the data are also consistent with the possibility that Ets family members collaborate with GATA-2 to reinforce Scl expression in a positive-feedback loop.

The similarities between the regulation of Scl and Gata2 are also underscored by the similarity of their functions in the hematopoietic system. Targeted mutation of both genes results in defects in both primitive and definitive hematopoiesis, resulting in mid-gestational lethality (Shivdasani et al., 1995; Tsai et al., 1994). This phenotypic similarity has not been demonstrated in the vascular system, where SCL has been shown to play a prominent role in vascular remodeling, whereas GATA-2 has not. However, because the vascular defects in Scl-null mice were only revealed after selective rescue of hematopoiesis, it seems likely that the early lethality of Gata2-null mice precludes a more precise analysis of the function of GATA-2 in the vascular system. Experiments are underway to circumvent the embryonic hematopoietic lethality and explore possible functions of GATA-2 in the vasculature.

The presence of multiple GATA-binding motifs within the VE enhancer also raises the issue of whether this enhancer might be auto-regulated by GATA-2, or even by another GATA factor. Of specific interest here, Grass et al. recently identified this same
element through its evolutionary sequence conservation during an analysis of GATA-1 regulation of the Gata2 gene in erythroid cells (Grass et al., 2006). In that study, the authors demonstrated that sequences overlapping the VE element exhibited robust activity in transfected erythroid cells and that elimination of the GATA sites abrogated the erythroid enhancer activity. Here, we show that the VE element is at least equally as active and as specific for endothelial cells in a rigorous in vivo assay. Whether the activity identified by Grass et al. and the activity we defined here represents an endothelial-enhancer activity that can simply be surreptitiously activated in erythroid cells or whether the element represents one that can be a bona fide target for GATA-factor activation in both hematopoietic and endothelial cells (as one might imagine for a hemangioblast-responsive element) awaits resolution following further investigation.

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